SYNOPSIS OF THESIS

METABOLIC REGULATION OF BLOOD PROGENITOR HOMEOSTASIS BY TCA CYCLE FOR DROSOPHILA LARVAL DEVELOPMENT

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SYNOPSIS

Chapter 1: Introduction

Haematopoiesis in *Drosophila melanogaster*

Haematopoiesis, the process of blood cell development, is a fundamental biological phenomenon conserved across metazoans. *Drosophila melanogaster*, a model organism, has contributed significantly to our understanding of haematopoiesis due to its genetic tractability and developmental simplicity. Despite differences in complexity between *Drosophila* and vertebrates, the fundamental principles and genetic pathways regulating blood cell development are remarkably conserved, making *Drosophila* an ideal system for studying haematopoiesis. In *Drosophila*, haematopoiesis occurs in two waves; first one is called Embryonic or primitive Haematopoiesis occurs during embryonic stage 5 from the head mesoderm, which is marked by the expression of mammalian GATA homologue Serpent (Srp) (Lebestky et al., 2000; Rehorn et al., 1996; Tepass et al., 1994). The second wave or definitive haematopoiesis, initiates from lateral mesoderm derived from the anterior trunk segment during mid-embryogenesis give rise to a small population of cells which homes around the dorsal vessel/heart and will subsequently develop into a hematopoietic organ called Lymph gland (Holz et al., 2003; Rugendorff et al., 1994). The lymphoid lineage in Drosophila is found to be absent whereas the hemocytes present are comparative to the mammalian myoid (Akira et al., 2001; Cherry & Silverman, 2006; Hultmark, 1994; Kleino & Silverman, 2014). Prohemocytes are multipotent progenitors which give rise to three mature blood cell populations, functionally important for all stages of life, namely plasmatocytes, crystal cells and lamellocytes (Evans & Banerjee, 2003; Hartenstein, 2006; Lanot et al., 2001; Lemaitre et al., 1996)**.**

TCA cycle and its variations

Mitochondria is regarded as an organelle of extreme importance to the cell as it is considered as the powerhouse of the cell, as it is the main site for ATP production in the oxidative respiration. But mitochondria have much more to its credit than this; the usual notion is that mitochondria execute its activity under the nuclear command, however it

is now established that its rather bidirectional regulation. For complex and high energy functions (eg. differentiation, stress adaptation, etc.) cells have to take mitochondrial fitness into account. Also mitochondria can influence nuclear genes to regulate different cellular functions (Chandel, 2015). Cellular respiration is a food web of biochemical reactions that fuels the growth and survival of the cell which in turn supports the organism, through three major outputs; energy (ATP), reducing equivalents (glutathione, NADH, FAD, etc.), and macromolecular precursors (Amino acids, nucleotides, simple sugars). Conventionally TCA cycle begins with the condensation of OAA (4-carbon) with acetyl CoA (2-carbon) to generate citrate (6-carbon). Subsequent reactions oxidize citrate to generate two CO_2 molecules, one GTP, four reducing equivalents (three NADH and one $FADH_2$) and finally regenerate the OAA. (Krebs & Eggleston, 1940; Ryan & O'Neill, 2020). But there are versions of TCA cycle reported across the literature in different cell states, eg. conversion of pyruvate to mt-OAA and glutaminolysis (glutamine to α-KG, through glutamate) in the events of citrate being exported to cytosol; glutamine dependent reductive carboxylation, here the cycle runs backward from glutamine derived α-KG to produce citrate, due to non-functional ETC (Lussey-Lepoutre et al., 2015; Metallo et al., 2011; Mullen et al., 2011). Citrate-malate shuttle is being utilised by cancer cells, Immune cells as well as ESCs to send the citrate out in the cytosol, where it is broken down with the help of ATP Citrate Lyase (ACL) into OAA and acetyl CoA, followed by cytosolic-OAA reduced to malate and transported back to the mitochondria. Another scenario where non-canonical TCA cycle can come into play is when oxygen cannot be accessed as terminal electron acceptor, due to ETC inhi bition, SDH complex reduces fumarate to succinate and fumarate acts as the electron acceptor to adapt the cell in oxygen limiting conditions (Bisbach et al., 2020; Chouchani et al., 2014; Spinelli et al., 2021).

Similar to above mentioned scenarios our work also highlights an interesting non-canonical version of TCA cycle which takes place in the Drosophila larval lymph gland development where only a few steps are found to be necessary, in comparison to the progenitor maintenance, where almost all the steps (CS to Sdh) are found to be important. Our work also brings out the importance of citrate in the early development of lymph gland by regulating progenitor and non-progenitor proliferation and keeping a check on the differentiation.

The current study titled **'metabolic regulation of blood progenitor homeostasis by TCA cycle for Drosophila larval development'** is divided into six chapters. The first

chapter highlights and reviews the literature on *Drosophila* haematopoiesis and TCA cycle and its variants, as well as discusses open questions. The second chapter describes the objectives of the study highlighting the role of TCA cycle in the development of lymph gland as well as maintenance of progenitor population in lymph gland. The third chapter deals with material and methods of the thesis, *Drosophila* genetics, immunohistochemistry, confocal imaging and metabolite supplementation to achieve the objectives of the thesis. Chapter four discusses the findings of the thesis and is divided into four main parts. Chapter four (Part 1) of the thesis, highlights the importance of TCA cycle for the maintenance and development of blood progenitors in lymph gland. Chapter four (Part 2) deals with the disconnect between aconitase and α-kdh in the early development of lymph gland. Chapter four (Part 3) traces the developmental defect of lymph gland growth by temporal analysis in different mutants (CS, mAcon and α-kdh). Chapter four (Part 4) establishes Citrate in progenitor compartment is necessary early in the development for the growth of lymph gland. The Fifth chapter highlights future directions and our contribution to current understanding of similar mechanisms during blood-progenitor development in both invertebrates and vertebrates.

Chapter 2: Objectives

This thesis work describes **"metabolic regulation of blood progenitor homeostasis by TCA cycle for Drosophila larval development",** highlights that the TCA cycle controls the development, maintenance and differentiation of progenitors in lymph gland. For the development part, we have identified that only a few steps (OAA to Succinyl CoA) not the whole TCA cycle regulates the lymph gland development. Whereas the whole cycle, except for *Fum* and *Mdh*, contribute for the maintenance of progenitor population. Which propose the idea that a variant of TCA cycle with a few steps comes up first during the development, which later on completes as a full cycle regulating the maintenance and differentiation of blood cells.

The specific objectives of this thesis are described below:

1. To understand the importance of TCA cycle in the development, maintenance, heterogeneity and differentiation of blood progenitors
2. To understand the metabolic aspect of LG size defect through temporal analysis in progenitor population

Chapter 3: Materials and Methods

Various Drosophila stocks used in this study were obtained from Vienna Drosophila Resource Centre (VDRC), Bloomington Drosophila Stock centre (BDSC) or were requested from the different research groups. All fly stocks were reared on corn meal agar medium with yeast supplementation at 25°C incubator unless specified. The crosses involving RNAi lines were maintained at 29ºC to maximize the efficacy of the Gal4/UAS RNAi system. Controls refers to either w1118 (wild type) or Gal4 drivers crossed with w1118. For staining blood cell progenitors, 3rd instar larval lymph glands were dissected out in 1X PBS and fixed in 4% formaldehyde solution, for 15 min. Post-fixation the tissues were permeabilized by 3 washes of 15 min each in 0.3% PBST (0.3% Triton-X in 1X PBS). Tissues were then blocked in 5% NGS (Normal Goat Serum), followed by overnight primary antibody treatment. Next day tissues were washed (as earlier) and incubated in secondary antibodies for 2-3 hours, followed by washing (as earlier) and mounting in mounting media. Immunohistochemistry on lymph gland was performed with the following primary antibodies: mouse α-P1 (1:30, I. Ando), rabbit α-Pxn (1:1000, J. Shim), mouse α-Myo (1:100, DSHB), rabbit α-PPO (1:200, H. M. Müller), and α-DHE (1:1000, Life technologies), rabbit α-pH3 (1:100; CST 3642S), rabbit α-Caspase 3 (1:200; CST 9661S). All the secondary antibodies were used at 1:500 dilutions, those are FITC, Cy3 and Cy5 (Jackson Immuno Research Laboratories) and Alexa Fluor 488, 546, 647 (Invitrogen). Nuclei were visualized using DAPI/Hoechst-33342 (Sigma B2261). Samples were mounted in mounting media (Vectashield, Vector Laboratories). Immuno-stained images of lymph glands were acquired using Olympus FV3000 confocal microscopy on 40X oil-immersion objective. Image was scanned with a frame size of 800 X 800 with 0.5/1/2um thickness for Z-stack, depending upon the experiment. All images were quantified using ImageJ software (NIH, USA). For that roughly, middle two confocal Z-stacks were merged and area was selected according to the respective staining and measured with the help of freehand selection tool of ImageJ software, followed by using the “measure tool” under “analysis tool” to get the area values. This was done for respective zones, where Tep4 or Dome area represents the progenitor zone, Dome/Pxn area represent differentiating or intermediary zone, Pxn/P1 and Hml/P1 area represent differentiated zone. The areas were represented in percent values with respect to the total area of the respective lobe. Controls were analysed in parallel to the tests every time. A minimum of 10-15 animals were analysed each time and the experiment was repeated at least three times. The quantifications represent the

mean (% area) of the independent experimental sets. Crystal cell population, as well as Caspase 3+ cells were analysed by counting total number of cells across the Z-stacks by merging all the stacks of lymph gland. These cell counts are represented as the total number of cells per lymph gland lobe. To count the total nuclei, we used the ImageJ software (NIH, USA). All the stacks of the lymph gland were merged with the help of "Z stack tool" and the primary lobe was outlined by the freehand selection tool, followed by "clear outside tool" under "edit option" to remove the other tissues (e.g. ring gland, secondary, tertiary lobes, dorsal vessel etc.). The nuclei of the remaining primary lobe were then threshold using "threshold tool" under "Image-adjust option" to obtain 8-bit image in single channel. The image obtained is then subjected to "watershed tool" under "Process-Binary option" followed by "particle analysis" with particle size 3-infinity and circularity of 0.04-1.00. This gave the total number of particles (nuclei) which were marked by Hoechst dye, in blue channel. Similarly, for Dome+ nuclei only green channel was subjected to this mentioned processing and for the Dome- nuclei $Dome^+$ nuclei were subtracted from total nuclei for the respective lobes. Total $pH3^+$ cells were counted by merging all the Z-stack together to give total number of $pH3^+$ cells in the entire lobe. For assessing $pH3^+$ population in $Dome^+$ zone, domeGFP channel was overlaid onto pH3 channel and for the $Dome^-$ $pH3^+$ cells $Dome^+$ $pH3^+$ cells were reduced from total pH3+ cells. Supplemented food was prepared by adding metabolites (1% citrate and 3% succinate; weight/volume) to the regular fly food. Eggs or larvae of respective stages were transferred to the supplemented food according to the experimental requirements for lymph gland analysis. In early feeding only the larvae were transferred back to the regular food after rearing on supplemented food for further development. Controls for the respective experiments were always treated similarly. All statistical analyses were performed using GraphPad Prism 10 software and Microsoft Excel 2010. The medians were analyzed using Mann-Whitney test, two-tailed and means were analyzed with unpaired t-test, two-tailed. For all the experiments batch effects were plotted.

Chapter 4: Results and Conclusions

4.1 TCA cycle is necessary for the maintenance and development of blood progenitors in lymph gland

To investigate the influence of this metabolic cycling in the maintenance of progenitor homeostasis and development of lymph gland, we utilized the binary system approach (UAS-GAL4). We systematically knock down each step of the TCA cycle with the help of four different GAL4s, which were temporally segregated but at the same time overlapping to certain extent. The GAL4s used in the study are *Tep4-GAL4,UASmcherry* (Core or subset of progenitors*),* d*omeMESO-GAL4,UAS-GFP* (MZ)*, ChIZ-GAL4* (IZ)*, and Hml-GAL4,UAS-2X EGFP* (CZ) in the order of their temporal expression.

By perturbing the enzymes of TCA cycle individually in the progenitor compartment at wandering 3rd instar larval stage, with d*omeMESO-GAL4,UAS-GFP* drivers we observed two very different phenotypes arising independent of each other. One of the phenotypes was found to be related to maintenance of the progenitor pool and regulation of differentiation. Here we found that for the progenitor maintenance, the TCA cycle was found to be functioning together for almost its entire length except for *Fum* and *Mdh* steps, which did not show any progenitor loss or increase in differentiation profile. The loss of progenitor population was observed by loss of GFP positive area expressed by d*omeMESO-GAL4,UAS-GFP* compared to respective controls. For d*omeMESO-GAL4,UAS-GFP* driver the reduction in the progenitor population is either compensated by increase in the double positive ($Dome^+$ Pxn^+) population or the Pxn^+ only population, we also observed around 15% increase in the plasmatocyte population marked by P1. The crystal cells, marked by PPO were found to be significantly decreased in the *CS, mAcon1, Kdh, Skap* and *Sdha* whereas there was a significant increase in the crystal cells in the *Idh* step while no change was observed in the *Fum and Mdh2* steps. Lamellocytes were not observed by manipulating any step of the TCA cycle.

We also tried to understand the influence of the subset of early progenitors on the lymph gland with the help of *Tep4-GAL4,UASmcherry.* Upon knocking down individual steps of the TCA cycle in these progenitor subset, the loss of progenitor was observed in all the steps at wandering 3rd instar larval lymph gland. This loss of progenitor population was found to be compensated by Tep4- Pxn- population, which is speculated to be $Dome^+$ only population, as no significant changes were noted in the Pxn+ population. No significant change was observed in the plasmatocyte population ($P1^+$)**,** whereas crystal cells, marked by PPO were found to be significantly decreased in the *CS,*

mAcon1, Kdh, Skap and *Sdha* whereas there was a significant increase in the crystal cells in the *Idh* step while no change was observed in the *Fum and Mdh2* steps.

Also to understand whether this regulation on progenitor maintenance is autonomous or non-autonomous, we also perform the knock down of TCA in the differentiating population with the help of *ChIZ-GAL4* (IZ), and *Hml-GAL4,UAS-2X EGFP* (CZ) drivers marking respective zones. *ChIZ-GAL4* marks the IZ which is an overlapping population of MZ and CZ ($Dome^{+}$ Hml^{+}). Manipulating the TCA cycle in this compartment does not lead to any increase in the differentiating population area (Pxn^{+}) which indirectly highlights that there is no feedback from this population to the progenitor maintenance involving TCA cycle. In case of CZ marked by *Hml-GAL4,UAS-2X EGFP,* we did not observed any change in the either of the differentiating population (Hml^{+} area or Pxn^{+} area), this further strengthens the results from *ChIZ-GAL4* and highlights the fact that the progenitor homeostasis is the autonomous function of the progenitor compartment through TCA cycle.

The other phenotype observed due to the knockdown of TCA cycle genes in the progenitor compartment was a growth defect of the lymph gland. With d*omeMESO-GAL4,UAS-GFP* driver we observed the lymph gland growth defect at *CS, mAcon1, and Kdh* steps whereas with *Tep4-GAL4,UASmcherry* driver the growth defect was observed at *mAcon1 and Kdh* only highlighting two important aspects; firstly *CS* is not necessary in the Tep4 compartment of the growth and second is that TCA does not run as a cycle early in development at least for the growth of lymph gland as the *Idh* step which connects *mAcon1* with *Kdh* does not show growth defect in any of the progenitor compartments. Similar results were seen in the IZ where growth defects were observed in *mAcon1, Idh and Kdh,* suggesting the coming together of the whole cycle whereas in CZ growth defect were observed in *CS, mAcon1, and Kdh* steps.

4.2 What fuels Kdh for early development of lymph gland in progenitors

The next question we asked was if not CS and mAcon1, what fuels the Kdh enzyme? From the available literature we got to know that the Gdh can also work reversibly and convert the glutamate into alpha-keto Glutrate. We first tested Gdh for the progenitor loss, differentiation profile and growth defect at 3rd install larval stage as was done for all other steps. We found that Gdh^{RNAi} recapitulated all the phenotypes of the *Kdh* knockdown from decrease in progenitor population, increase in double positive

(Dome^{+}Pxn^{+}) population, increase in P1 population, decrease in PPO^{+} crystal cells to growth defect. This convinced us to believe that Kdh was fueled by Gdh in the early development of LG. We further did the temporal analysis of the Gdh and found that similar to the Kdh, the growth defect was observed in Gdh at 48h itself, both Dome^{+} and Dome^{-} compartments were equally compromised similar to Kdh and also the mitotic activity (pH3) was also equally compromised in both the compartments. These results further validate the connection between Kdh and Gdh.

4.3 Temporal analysis of lymph gland development in CS, mAcon and kdh knockdowns

The growth defect phenotype was common amongst all three *CS^{RNAi}*, *mAcon1^{RNAi}* and *Kdh^{RNAi}* mutants, at wandering 3rd instar larval stage. It could be due to two different scenarios: a) due to cell death (apoptosis) and b) due to non-proliferation (cell cycle arrest). We first explored the apoptosis possibility; where we stained the wandering 3rd instar larval lymph gland with Cleaved-Caspase 3 antibody to mark apoptotic nuclei in all the mutant backgrounds with growth phenotype. We used *Hml$^{\Delta}$-Gal4, UAS-GFP; UAS-Hid* as a positive control for Hid being activator of apoptosis, Caspase 3 puncta were observed around the nuclei. Whereas the mutants in study did not show any considerable change in comparison to the positive control, which negated apoptosis as a reason for size defect.

To understand the lymph gland growth defect due to knockdown of certain steps of TCA cycle we tracked the development of lymph gland temporally at 48h, 72h, 96h and 120h respectively. For this study we utilized the progenitor specific d*omeMESO-GAL4,UAS-GFP.* Upon temporal analysis we found both *CS^{RNAi}* and *mAcon1^{RNAi}* had shown growth defect at 96h whereas *Kdh^{RNAi}* had shown growth defect at the 48h time point itself; growth defect was analysed by counting the total number of nuclei per LG lobe in the mutants compared to the respective controls. When we further analysed the Dome^{+} vs Dome^{-} population in the respective lobes, we observed that the Dome^{+} population was found to be reduced in both *CS^{RNAi}* and *mAcon1^{RNAi}* at the 72h itself highlighting the fact that the growth defect arising at the 96h is the result of reduction in the Dome^{+} (Progenitor) population. Growth defect was not observed at the 72h itself due to the compensatory effect of Dome- (non-progenitor) population, but this population also could not compensate for long and therefore growth defect was observed at the 96h.

Whereas in Kdh^{RNAi} the defect was equally noticed in both Dome^{+} as well as Dome- compartments from the 48h itself.

To understand why the proliferative capacity of the Dome^{+} (progenitors) cells is compromised we checked the differentiation profile of the LG temporally with the help of differentiation markers Pxn and P1. To our surprise in both CS^{RNAi} and $mAcon1^{RNAi}$ mutants we observed precocious differentiation, as we saw Pxn and to certain extent P1 positive cells at 48h itself, whereas the same wasn't observed for the Kdh^{RNAi} mutant. This was an important finding as here the cells were found to be dividing to differentiate rather than dividing to proliferate and this could be the major reason for CS and mAcon1 enzyme knockdown to end up in small LG. These results also highlight another important aspect that Kdh enzyme doesn't function in sync with the CS and mAcon1 even though all of them had the same end phenotype (growth defect). Also knockdown of *Idh, which* is a connection between these enzymes, in TCA cycle, do not show any growth defect, this further supports the fact that Kdh is functioning independent of both CS and mAcon1 at least early in development for the growth of LG.

4.4 Citrate in progenitor compartment is necessary early in the development for the growth of lymph gland

Since loss of CS and mAcon1 led to the growth defects we next tried to rescue the phenotype by supplementing the affected/ lost metabolite. We started with the supplementation of the product of CS (Citrate). With the help of available literature and screening we standardize the concentration of citrate to be 1% weight/volume. We transferred the embryos to 1% CF and allowed them to grow and dissected the LG at 3rd instar larval stage (120h). To our surprise the CS mutant LG further reduced and even control LG reared on 1% CF, compared to their own replicates on NF (Normal fly food), had shown the growth defect, since the larvae were reared completely on the supplemented food, the defect might be due to excess of citrate, which can also act as a stress signal. We then divided the larval growth trajectory into two parts based on the timing of onset of differentiation i.e., pre-differentiation and post-differentiation phase separated at 60h AEL time point. We next transferred the 2nd instar larvae at 60h AEL onto the 1%CF and allowed it to develop and at wandering 3rd instar (120h) dissected the LG for the analysis. Here also we could not rescue the growth defect. Finally, we transferred the embryos to the 1% CF and at 60h AEL (Pre-differentiation phase), they were again transferred back to the NF for further development and again at 120h of

development the LG were dissected out and this time we saw growth defects being rescued. We also confirmed the rescue of the progenitor homeostasis with the help of Pxn and P1 in 1% CF supplementation till 60h AEL. These results highlight the importance of citrate in early development of lymph gland.

Chapter 5: Discussion

With the provided data we have shown that early in development TCA cycle does not behave cyclic rather act as discrete set of steps regulating growth non-autonomously (*CS* and *mAcon1*) as well as autonomously (*Kdh* and *Gdh*). Here *CS* and *mAcon1* non autonomously promote progenitor proliferation by inhibiting the differentiation as well as checking the proliferation of non-progenitor population. This indirectly helps the *Kdh* and *Gdh* to promote progenitor proliferation autonomously. We have established the requirement of the citrate in the early development (upto 60h) of the lymph gland with the help of 1% citrate supplementation in the food. Later around the 60h AEL it was found that Idh bridges the gap between mAcon1 and Kdh, which also coincides with the time developmentally where the differentiation kicks in, and with our results too, we found that TCA becomes important for the maintenance of the blood progenitors by keeping a check on differentiation.

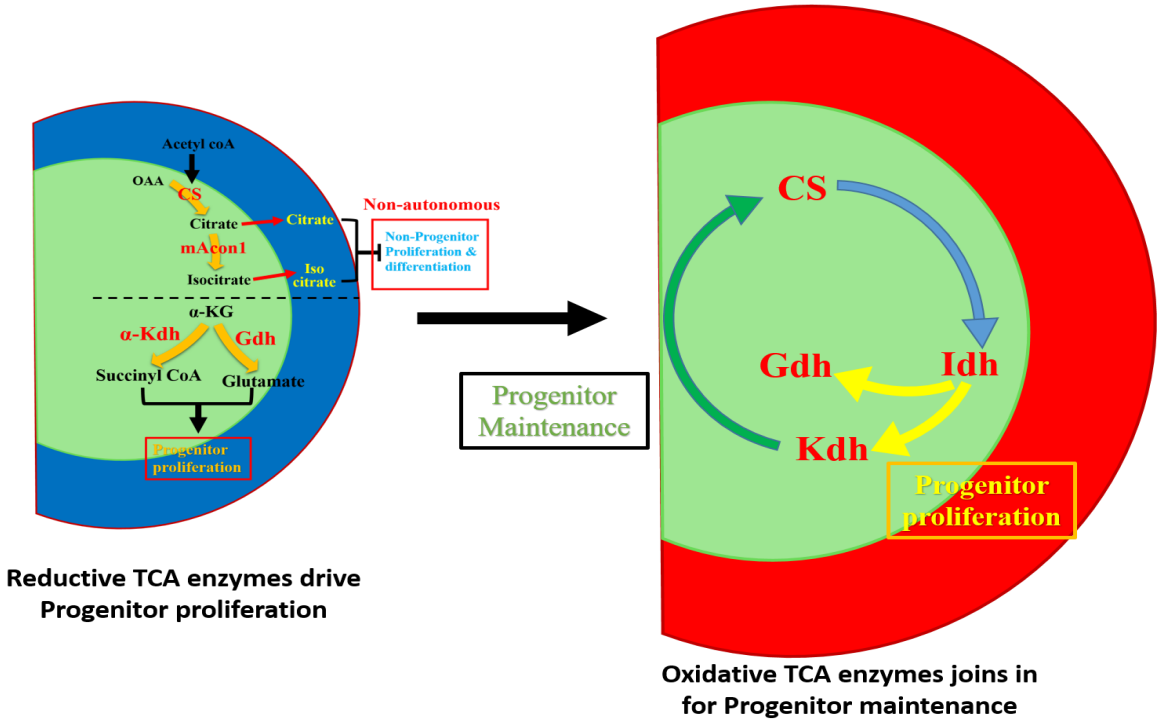

Figure 1. **Group of discrete steps regulating lymph gland growth to the cyclic TCA for the maintenance of blood progenitors.** The model describes the importance of TCA cycle enzymes regulating growth and progenitor maintenance in lymph gland. All

enzymes that promotes growth are shown in red, whereas their metabolites are shown in black. The first picture shows the transition from discrete steps important for growth in early development (till 60h AEL) to cyclic TCA important for progenitor maintenance post 60h AEL in the second picture. The green zone in the lymph gland cartoon represent the progenitor zone (MZ), blue zone represents the differentiating population (IZ) and the red zone represent the differentiated zone (CZ).

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LIST OF PUBLICATIONS DURING PhD

1. Kapoor A, **Tomar A***, Mukherjee T. (2023). Pumping up the blood progenitors by Piezo. Proc Natl Acad Sci U S A. 2023 Jun 6;120(23): e2306004120. doi: 10.1073/pnas.2306004120. (*Co- First author)
2. Goyal, M.**, Tomar, A.**, Madhwal, S., & Mukherjee, T. (2022). Blood progenitor redox homeostasis through olfaction-derived systemic GABA in hematopoietic growth control in *Drosophila*. Development (Cambridge, England), 149(8), dev199550. https://doi.org/10.1242/dev.199550
3. Preethi P, **Tomar, A.**, Madhwal, S., & Mukherjee, T. (2020). Immune Control of Animal Growth in Homeostasis and Nutritional Stress in Drosophila. Frontiers in Immunology. https://doi.org/10.3389/fimmu.2020.01528

LIST OF Ph.D. WORK PRESENTATION IN CONFERENCES

1. Poster presented at International Society for Stem Cell Research (ISSCR 2024), Hamburg, Germany (10-13 July 2024)
2. Poster presented at 19th International Congress of Developmental Biology by ISDB, Algarve Congress Centre, Herdade dos Salgados, Guia, Portugal (16-20 Oct 2022)
3. Poster presented at International Society for Stem Cell Research (ISSCR 2022), San Francisco (Virtual) (15-18 June 2022)
4. Poster presented at Indian Drosophila Research meeting (InDRC), by Indian Institute of Science Education and Research (IISER), Kolkata (Virtual). (13-17 Dec 2021)